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Emergence of IFN-lambda as a Potential Antitumor Agent

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1. Introduction

Despite the early discovery of interferon (IFN) in 1957, some members of the IFN family were just identified during the recent years. Interferon was discovered and characterized by Isaacs & Lindenmann during their study of viral infection and the biology of interference (Isaacs and Lindenmann, 1957). The authors used chick membranes infected with influenza viruses, and found that those cells released into the medium a substance which rendered other cells resistant to viral infection. The authors named this substance interferon. Subsequent studies demonstrated that interferon is not a virus particle but a protein released by the cells during viral infection.

Early studies have defined three different subfamilies of interferons, depending on their cell origin (Stewart et al., 1973). IFN-α was characterized from virus-infected leukocytes, IFN-β from fibroblasts and IFN-γ produced by transformed lymphocytes, which was first designated as an immune interferon. Other properties such as hydrophobicity, antigenicity and heat/pH sensitivity were also investigated to distinguish between IFN molecules. By probing on several hydrophobic adsorbents, one group demonstrated that the fibroblast interferon is more hydrophobic than leukocyte interferon (Jankowski et al., 1975). Rabbit antiserum prepared against the leukocyte IFN was found to contain two populations of neutralizing antibodies specific for leukocytes and fibroblasts populations and the authors concluded that two antigenic species of IFN were present (Havell et al., 1975). However, this rabbit antiserum preparation was shown to be less active against immune interferon (IFN-γ), which was also found to be relatively unstable at pH 2 and at 56 degrees (Valle et al., 1975). In the last 30 years, IFN-α, β and γ were purified and receptor binding assays using radio-labeled ligands were performed. The data clearly indicated that IFN-α and IFN-β interacted with the same binding site, which was distinct from IFN-γ (Littman et al., 1985; Merlin et al., 1985). Subsequently, characterization of the IFN receptor, followed by the production of IFN knockout mice, clearly showed that IFN-α/β signal through a receptor that is completely distinct from IFN-γ receptor (Ding et al., 1993; Muller et al., 1994). As a result of all these studies, IFNs were classified as two types. Type I IFN family is composed of several members, which include IFN-α, IFN-β and other related IFNs such as IFN-ω, IFN-ε and IFN-κ. The type II IFN family only includes IFN-γ (Pestka, 2007). In 2003, another

IFN family (type III) was identified and its members designated as IFN-λ (Kotenko et al., 2003; Sheppard et al., 2003) (Figure 1).

The new IFN members identified in human were designated as IFN-λs, by Kotenko's group (Kotenko et al., 2003), or IL-28A, IL-28B and IL-29 by Sheppard and coll. (Sheppard et al., 2003). IFN-λs demonstrate structural features that are similar to the IL-10-related cytokine family II [CRFII] but possess antiviral activity (Langer et al., 2004; Pestka et al., 2004). In 2005, the International Community of Interferon and Cytokine Research designated IFN-λs as type III IFNs, which include three distinct IFN-λ proteins, called IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). The genes encoding all three type III IFNs are clustered on human chromosome 19. The IFN-λ3 gene is transcribed in the opposite direction to the IFN-λ1 and IFN-λ2 genes. The coding region of each of the genes is divided into five exons (exon 1-5). The overall intron/exon structure of the IFN-λ genes correlates well with the common conserved architecture of the genes encoding IL-10-related cytokines (Kotenko, 2002). It is thought that the human IFN-λs genes derived from a common predecessor fairly recently. This is based on the fact that there is a great deal of homology between human IFN-λs It is also suggested that during the divergence of the IFN-λ1 and IFN-λ2 genes, occurred a more recent duplication event in which a fragment containing the IFN-λ1 and IFN-λ2 genes was copied and integrated back into the genome in a head-to-head orientation with the IFN-λ1-IFN-λ2 segment. It is speculated that this duplication may have created the IFN-λ3 gene, which is nearly identical to the IFN-λ2 gene in terms of the upstream and downstream flanking sequences and coding region. Therefore, the promoters of the genes for IFN-λ2 and IFN-λ3 share a great similarity and have many common elements with the IFN-λ1 promoter (Kotenko et al., 2003; Sheppard et al., 2003). Based on this, it is suggested that the IFN-λ genes are regulated in a similar fashion.

The members of this new IFN family were found to interact through unique receptors that are distinct from type I and type II IFN receptors. The receptor for type III IFN is composed of the unique IFN-λR1 chain and the IL-10R2 chain, which is shared with IL-10, IL-22 and IL-26 receptor complexes. Although type III IFNs bind to a specific receptor, the downstream signaling is similar to that induced by type I IFNs. Both type I and type III IFNs stimulate common signaling pathways, consisting of the activation of JAK1 and TYK2 kinases and leading to the activation of the IFN-stimulated gene factor 3 (ISGF3) transcription complex. ISGF3 is composed of STAT1 and STAT2, and the interferon regulatory factor IRF9 (ISGF3-γ or p48).

This complex translocates into the nucleus and interacts with a specific DNA sequence designated IFN stimulated response element (ISRE), present upstream of the genes stimulated by the IFNs. The Type II IFN activates cell signaling through another pathway. After the interaction with IFNGR, JAK1 and JAK2 are activated and phosphorylate STAT1, which dimerizes, translocates into the nucleus, binds to the gamma activated sequence (GAS) and induces gene expression.

Although there are three genes encoding highly homologous but distinct human IFN-λ proteins (IFN-λ1, IFN-λ2, and IFN-λ3), our search of the mouse genome revealed the existence of only two genes, representing mouse *IFN-λ2* and *IFN-λ3* gene orthologues, located in chromosome 7 and encoding intact proteins. The mouse *IFN-λ1* gene orthologue is a pseudogene containing some variations in addition to a stop codon in the first exon and does not code for an active protein (Lasfar et al., 2006). We have cloned the mouse IFN-λs

(mIFN-λ2 and mIFN-λ3) and IFN-λ receptor (mIFN-λR1) orthologues and found them to be quite similar to their human counterparts. Experiments showed that similar to their human counterparts, mIFN-λ2 and mIFN-λ3 signal through the IFN-λ receptor complex, activate ISGF3, and are capable of inducing antiviral protection and MHC class I antigen expression in several cell types. The results showed that murine type III IFNs (IFN-λs) engage a unique receptor complex, composed of IFN-λR1 and IL-10R2 subunits, to induce signaling and biological activities similar to those of type I IFNs. Interestingly, in contrast to type I and type II IFNs, type III IFNs demonstrate less specie specificity. This characteristic of type III IFN may be of prime importance in the development of a xenogenic model.

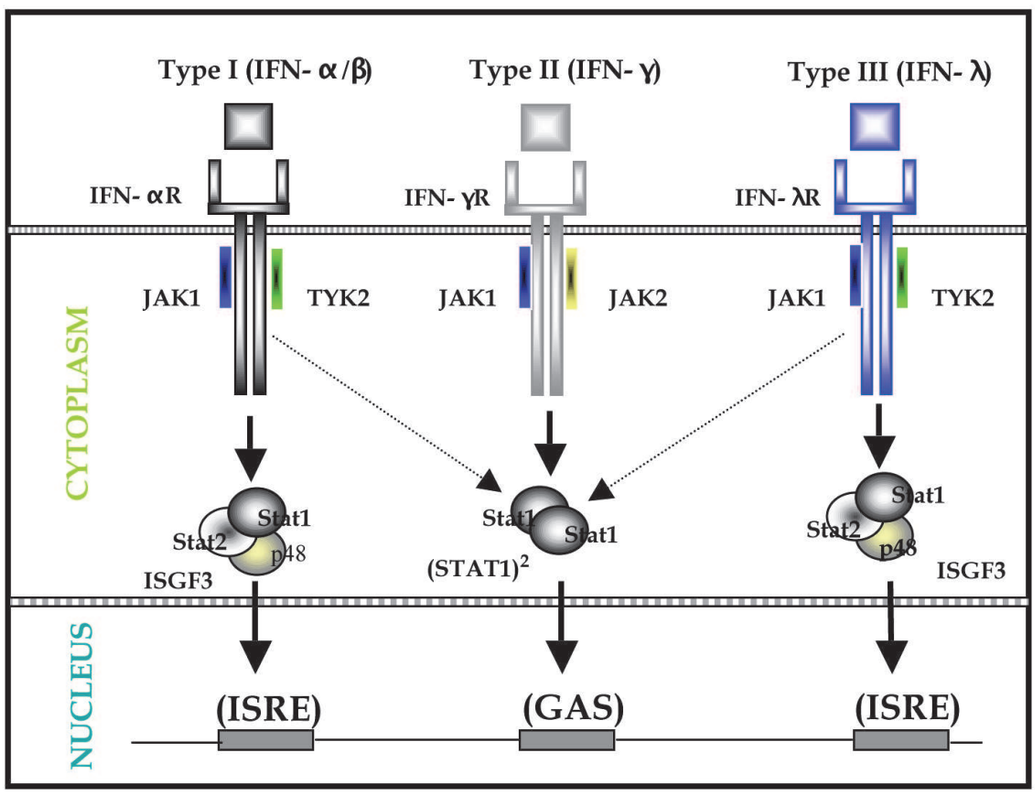

Fig. 1. Interferons, interferon receptors and cell signaling.

2. Characteristics of the IFN- λ receptor

2.1 The human IFN-λ receptor (hIFN-λR1)

The human IFN-λR1 (hIFN-λR1) consists of 520 amino acids, including a signal peptide of 20 amino acids. In SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis), the IFN-λR1 protein was estimated at around 70 kD, much higher than the theoretical molecular weight calculated at 56 KD, implying the existence of post-translational modifications (Witte et al., 2009). Although the extracellular domain presents 4 putative N-linked glycosylation sites and 1 O-linked glycosylation site, effective IFN-λR1 glycosylation has not been definitely established. The intracellular domain of IFN-λR1 contains three

tyrosine residues. Tyrosines 343 and 517 are essential for STAT2 and STAT5 activation (Dumoutier et al., 2004). The existence of splice variants of IFN-λR1 has been reported. Shepard and coll. suggested the presence of one splice variant lacking a part of exon VII (Sheppard et al., 2003). However, we did not confirm yet the existence of these splice variants. Another splice variant lacking exon VI was first described in 2003 (Dumoutier et al., 2004), and its existence has been recently confirmed. This variant was designated sIFN-λR1 for soluble IFN-λR1 or sIL-28R1 for soluble IL-28R1 (Witte et al., 2009). The cloning and protein expression analysis of the soluble IFN-λR1, sIFN-λR1, were performed and the ligand binding studies showed the aptitude of this soluble receptor to inhibit the IFN-λ response. However, high concentrations of sIFN-lR1 were used and only partial inhibition was achieved, suggesting that the described form of sIFN-λR1 may not play an important role in the regulation of IFN-λ response. However, we cannot rule out the induction of other post-translational modifications of the IFN-λR1 that may modulate the activity of sIFN-λR1 by increasing its inhibitory effects on circulating IFN-λs in normal or pathologic situations. Witte and coll. (Witte et al., 2009) showed the presence of sIFN-λR1 in all cells expressing IFN-λR1, with high levels in immune cells such as B, T and NK cells and suggested a correlation between the level of sIFN-λR1 and the lack of response to IFN-λ.

2.2 The mouse IFN-λ receptor and comparison to the human counterpart

After the identification of the human IFN-λ system, we cloned the mouse IFN-λR1 (mIFN-λR1) chain and found it around 67% similar to its human counterpart. The mIFN-λR1 is encoded on mouse chromosome 4D3. Although the mouse and human IFN-λR1 sequences are very similar, only two of three tyrosine residues of the human receptor intracellular domain are conserved in the mouse orthologue. In addition, the mouse receptor contains three additional tyrosine residues. There is also a stretch of negatively charged residues close to the end of the human receptor intracellular domain. This region in the mouse receptor is significantly altered by a short insertion and substitutions of several amino acid residues, resulting in a longer and more negatively charged region in the mouse receptor (18 of 20 amino acids are negatively charged). Two tyrosines, Tyr^{343} and Tyr^{517}, of hIFN-λR1 can independently mediate STAT2 activation by IFN-λs. Interestingly, the Tyr^{341}-based motif of mIFN-λR1 (YLERP) shows similarities with that surrounding Tyr^{343} of hIFN-λR1 (YIEPP). In addition, the COOH-terminal amino acid sequence of mIFN-λR1 containing Tyr^{533} (YLVRstop) is very similar to the COOH-terminal amino acid sequence of hIFN-λR1 containing Tyr^{517} (YMARstop). Therefore, both the mouse and human IFN-λR1 chains contain similar docking sites for STAT2 recruitment and activation, YΦEXP and YΦXRstop (where Φ is hydrophobic). Thus, Tyr^{341}- and Tyr^{533}-based motifs on mIFN-λR1 are also likely to mediate STAT2 recruitment and, therefore, mediate ISGF3 activation, which is responsible for most of the IFN-λ-induced biological activities. Interestingly, by using hamster cells transfected with chimeric human IFN-λR1/γR1 and IL-10R2 expression vectors, we demonstrated that the cells were responsive to both human and mouse IFN-λs, as measured by STAT1 activation in electromobility shift assay and up-regulation of MHC class I antigen expression (Lasfar et al., 2006). However, expression of murine IFN-λR1/γR1 alone rendered hamster cells responsive to both human and mouse IFN-λs, implying that hamster IL-10R2 can dimerize with murine IFN-λR1 to mediate signaling in response to either human or mouse IFN-λs. As controls, we did not detect any response of parental hamster cells to either human or mouse IFN-λ (Lasfar et al., 2006). Therefore, the mouse and human IFN-λs are not specie specific.

3. Distribution of IFN-λR1 and responsiveness to IFN- λ

The functional IFN-λR is formed by two chains, IFN-λR1 (also called IL-28R1) and IL-10R2. IFN-λR1 is unique for the IFN-λs and its tissue distribution is highly restricted. In contrast to IFN-λR1, IL-10R2 is shared by IL-10, IL-22 and IL-26 and ubiquitously expressed in all tissues. Unlike IFN-α, only few cell types respond to IFN-λ (Figure 2) . In contrast to the epithelial-like cells, fibroblasts and endothelial cells were completely unresponsive to IFN-λ (Lasfar et al., 2006). Although the hematopoeitic system is not the primary target of IFN-λ, the response of some subpopulations to IFN-λ is not excluded. In mice, we found that IFN-λ induces STAT1 activation in both plasmacytoid and myeloid dentritic cells (Abushahba et al., 2010). These results are in accordance with Mennechet and Uze (Mennechet and Uze, 2006), who proposed the acquisition of an IFNλ response by the monocytes after their differentiation into dentritic cells. Therefore, the response to IFN-λ may be controlled by the induction of the IFN-λR1 expression. Recently, Witte and coll. found different levels of IFN-λR1 in different tissues (Witte et al., 2009). The highest levels were found in the gastrointestinal tract and lung. The brain showed the lowest level. The IFN-λR1 expression

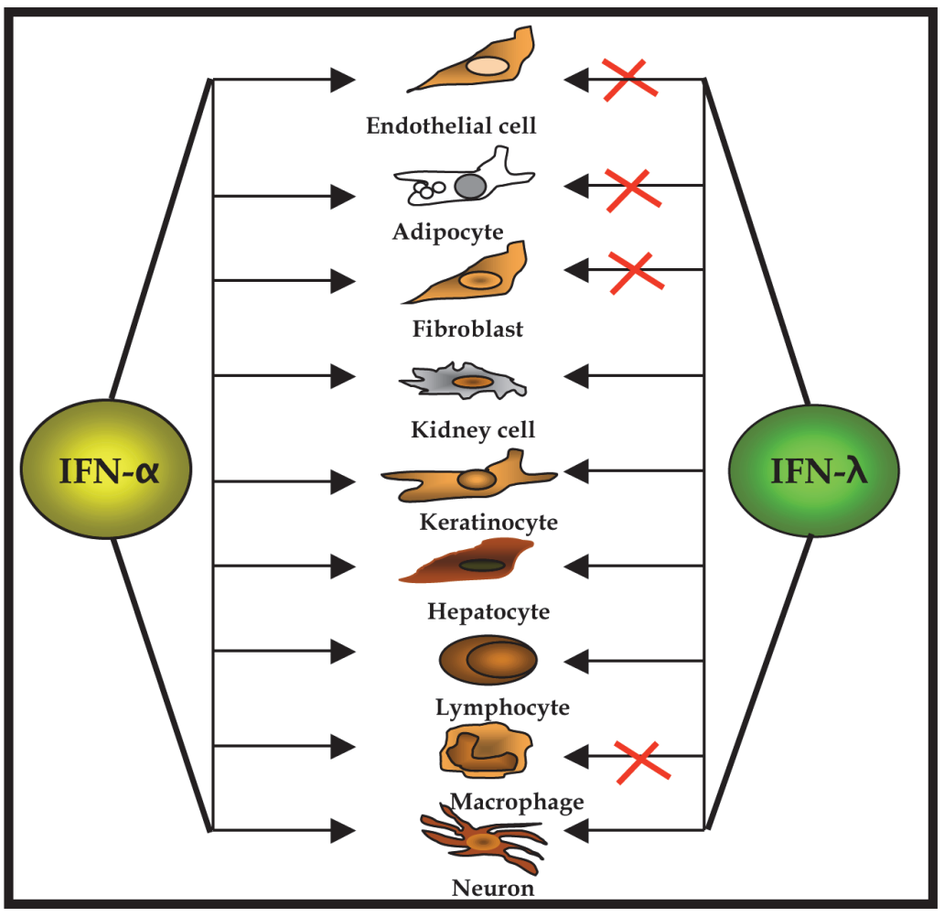

Fig. 2. Cellular targets for type I and type III IFNs. Cells from different origins were tested for IFN-α and IFN-λ response by measuring the IFN induced-cell signaling (Stat activation) and biological activity (MHC class I antigen stimulation).

was also analyzed in different cell types. The expression of cell populations isolated from human skin showed a high expression of IFN-λR1 in keratinocytes and melanocytes. However, dermal fibroblasts, endothelial cells and sub-dermal adipocytes did not express significant amounts of IFN-λR1. Significant expression of IFN-λR1 was detected in primary human hepatocytes in comparison with the condrocytes, isolated from the hyaline cartilage of the knee joint (Witte et al., 2009; Wolk et al., 2008). Although the expression of IFN-λR1 was significantly high in lymphoid tissues, the IFN-λ response was very weak, implying the presence of specific mechanisms on the lymphoid tissues that may inhibit the IFN-λ response. For example, IFN-λR1 levels in B cells are three fold those detected in keratinocytes, which exhibit one of the highest response to IFN-λ. Witte and coll. proposed the potential role of sIFN-λR1, highly released by the immune cells, in this weak response to IFN-λ (Witte et al., 2009).

Although all the IFN-λs interact with the same receptor, IFN-λR1, the binding characteristics for each ligand are still under investigation. In the future, it will be important to analyze the IFN-λ activity in the light of the IFN-λ binding to the cells and understand particularly the role of IFN- λ3, which possesses the highest activity as compared with the other IFN-λs (Dellgren et al., 2009). Analysis of the ligand binding in combination with the activity induced by IFN-λ will be also important in understanding the role of IFN-λ in epithelial cells, particularly in comparison with the immune cells expressing IFN-λR1. Besides several carcinomas, originating from epithelial cells, which respond to IFN- λ, other tumors not arising from epithelial cells may become more sensitive to IFN-λ. It was reported that multiple myeloma cells, which originate from B cell plasmocytes, showed high binding and response to IFN-λ (Novak et al., 2008). Studying the IFN-λ binding in transformed cells versus normal cells may be very helpful for tumor targeting and for the establishment of the optimum dose of IFN- λ to be used for the *in vivo* treatment. IFN-λ can also be used as a drug carrier, to specifically target a drug to tumors expressing high IFN-λ binding sites.

4. Biological and clinical activities of IFN-λ

4.1 Comparative studies between type I and type III IFN (IFN- λs)

To date, every cell line responding to IFN-λ also responded to type I IFNs. However, the cell signaling induced by type III IFNs appeared to be significantly weaker as compared to type I IFNs. Interestingly, the intensity of cell signaling induced by IFN-λ, as assessed by STAT activation, is not always correlated with the level of biological activity, as determined by MHC class I expression (Figure 3).

Antiviral studies performed *in vitro* and *in vivo* have shown that both IFN-α and IFN-λ contribute to the overall host antiviral defense system (Ank et al., 2008; Ank et al., 2006; Kotenko et al., 2003; Kugel et al., 2009; Mordstein et al., 2008; Sheppard et al., 2003). It has been demonstrated that IFN-λ induces antiviral activity against VSV (vesicular stomatitis virus) and EMCV (encephalomyocarditis) in many cell types (Kotenko et al., 2003; Li et al., 2009; Sheppard et al., 2003; Uze and Monneron, 2007). Several studies demonstrated that type III IFNs can also inhibit replication of Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) *in vitro* (Hong et al., 2007; Lazaro et al., 2007; Marcello et al., 2006; Robek et al., 2005; Uze and Monneron, 2007). These studies were important since they underlined the fact that IFN-λ could be used as an alternative to IFN-α for HCV patients who are resistant to IFN-α treatment. Just recently, it has been reported that IFN-λ has the ability to inhibit human immunodeficiency virus type 1 (HIV-1) infection of blood monocyte-derived macrophages

that expressed IFN-λ receptors (Hou et al., 2009). However, in most other cases, the antiviral potency of IFN-λ against several viruses seems to be lower than that of IFN-α (Kotenko et al., 2003; Li et al., 2009; Marcello et al., 2006; Meager et al., 2005; Mordstein et al., 2008; Sheppard et al., 2003). In addition, IFN-λ and IFN-α may induce distinct signal transduction and gene regulation kinetics (Maher et al., 2008; Marcello et al., 2006).

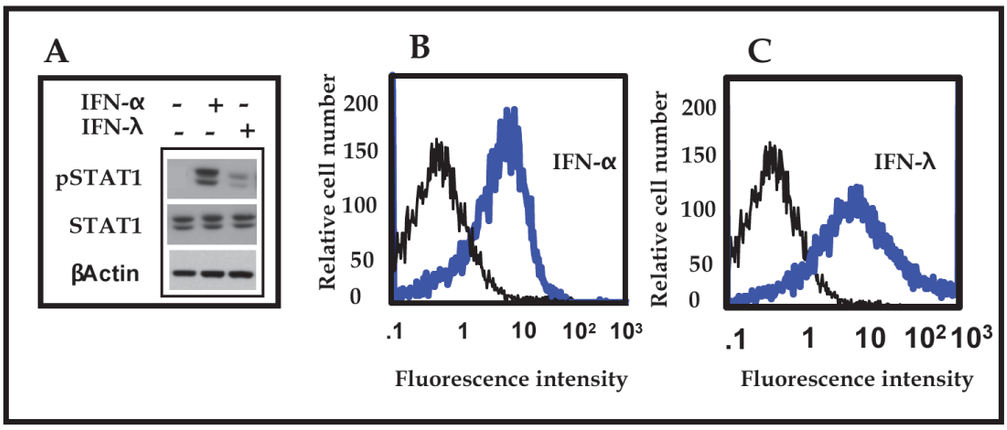

Fig. 3. Intensity of the IFN signaling and biological activity induced by IFN-α and IFN-λ. B16 melanoma cells were treated with IFN-α or IFN-λ followed by STAT1 activation (A) and MHC class I antigen expression (B and C) analysis.

Moreover, Type I IFN-α activates a plethora of innate and adaptive immune mechanisms that help eliminate tumors and viral infections. IFN-α immunoregulatory functions include major histocompatibility complex (MHC) class I expression in normal and tumor cells, activation of NK cells, dendritic cells (DCs) and macrophages, resulting in the promotion of adaptive immune responses against tumors and virally infected cells (Biron, 2001; Le Bon and Tough, 2002). The role of IFN-λ in the immune system is currently being investigated by several groups. So far, data suggests that IFN-λ exerts immunomodulatory effects that overlap those of type I IFN. It has been recently demonstrated that human IFN-λ1 (IL-29) modulates the human cytokine response (Jordan et al., 2007a). IFN-λ1 treatment of whole peripheral blood mononuclear cells (PBMC) up-regulated the expression of IL-6, IL-8, and IL-10 but not IL-1 or TNF. This IFN-λ-induced cytokine production was inhibited by IL-10. By examination of purified cell populations, it was also shown that IFN-λ1 activated monocytes and macrophages, rather than lymphocytes, resulting in the secretion of the above panel of cytokines, suggesting that IFN-λ1 may be an important activator of innate immune responses particularly at the site of viral infections (Jordan et al., 2007a). IFN-λ1 was also shown to possess immunoregulatory functions on T helper 2 (Th2) responses by markedly inhibiting IL-13. However, only moderate effect was observed on IL-4 and IL-15, the other important cytokines in the Th2 response. (Dai et al., 2009; Jordan et al., 2007b; Srinivas et al., 2008). This immunoregulatory function was enhanced through the expression of IFN-λR1 on $CD4^+$ T cells (Dai et al., 2009). These findings correlate with data suggesting that IFN-λ may have an immunoprotective role against asthma, the allergy disease caused by an exaggerated Th2 response (Bullens et al., 2008; Johnston, 2007; Li et al., 2009).

Similar to IFN-α, IFN-λ produced by DCs, in response to Toll-like receptor (TLR) stimulation, was found to have specific effects on DC differentiation and maturation (Coccia et al., 2004), which include only partial maturation of DCs, upregulation of MHC class I and

II molecules, and no induction of co-stimulatory molecules (Li et al., 2009; Mennechet and Uze, 2006). During their differentiation from monocytes, DCs acquire IFN-λ responsiveness through the expression of IFN-λR1. Interestingly, DCs treated with IFN-λ promoted the generation of tolerogenic DCs and the IL-2 dependent proliferation of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells (Tregs) (Mennechet and Uze, 2006). More recently, Morrow and coll. have demonstrated through DNA vaccination with plasmids encoding IFN-λ3 (IL-28B) and IL-12, that IFN-λ3, just like IL-12, is able to enhance adaptive immunity. However, in contrast to IL-12, IFN-λ3 reduces regulatory T-cell populations. They also showed that unlike IL-12, IFN-λ3 is able to increase the percentage of splenic $CD8^+$ T cells in vaccinated animals and that IFN-λ3 can completely protect mice from death following a lethal influenza challenge (Morrow et al., 2009). These studies altogether highlight the strong candidacy of IFN-λ as a potential novel immunotherapeutic agent.

In addition to antiviral and immunomodulatory activities, type I IFNs demonstrate antiproliferative activities in most cell lines, while this activity seems to be restricted with IFN-λs (Li et al., 2009; Meager et al., 2005). Type I IFNs have been shown to induce apoptosis in tumor cells. Yet, the molecular mechanisms mediating cell death in response to these IFNs remain to be fully explained. By binding to their corresponding cellular receptor complexes, IFNs induce a quick and potent signaling which leads to the expression of more than 300 IFN-stimulated genes (ISGs) (Der et al., 1998; Doyle et al., 2006; Marcello et al., 2006). Many ISGs encode proteins that have been implicated in apoptosis (Clemens, 2003; Kalvakolanu, 2004). Unlike IFN-α, IFN-λs do not inhibit the proliferation of several cell lines including Daudi cells (a B-lymphoblastoid cell line from Burkitt's lymphoma), which strongly respond to type I IFNs in an antiproliferative assay (Kotenko et al., 2003; Meager et al., 2005; Sheppard et al., 2003; Uze and Monneron, 2007). However, it was demonstrated that IFN-λs do inhibit the proliferation of few tumor cell lines, such as the LN319 human glioblastoma cell line (Meager et al., 2005) and of cells constitutively expressing high levels of IFN-λR1 (Dumoutier et al., 2004). The antiproliferative effects of IFN-λ have been demonstrated in various tumor cell lines that express ectopic or endogenous IFN-λ receptors (Brand et al., 2005; Meager et al., 2005; Zitzmann et al., 2006). Therefore, the ability of IFN-λs to induce antiproliferative activity in cells depends on the level of IFN-λR1 expression.

It has been reported that IFN-λ signaling in colorectal adenocarcinoma HT29 cells led to caspase activation, externalization of phosphatidylserine (PS), and DNA fragmentation, resulting in subsequent apoptosis (Li et al., 2008). This study provided evidence for the first time that type III IFNs, alone or in combination with other stimuli, have the potential to induce apoptosis. Moreover, another recent study revealed that IFN-α and IFN-λ differ in their antiproliferative effects and this was correlated with a difference in the duration of JAK/STAT signaling activity between the two IFNs and prolonged ISG expression upon IFN-λ treatment (Maher et al., 2008). Using the human keratinocyte HaCaT cell line that expresses receptors for both IFN-α and IFN-λ, they found that IFN-λ induced a more pronounced growth inhibitory effect than IFN-α. IFN-λ was also more efficient than IFN-α in inducing an antiproliferative effect that overlapped with the activation of apoptosis. Prolonged duration of IFN-λ-induced STAT activation and ISG expression could account for the enhanced antiproliferative and pro-apoptotic effects observed in HaCaT cells, effects not seen upon treatment with high doses of IFN-α (Maher et al., 2008). Interestingly, a study has shown that IFN-λ can induce the growth of multiple myeloma cells and antagonize the dexamethasone-induced cell death in these cells (Novak et al., 2008). IFN-λ-mediated cell growth of multiple myeloma cells was MAPK dependent (Novak et al., 2008). High level of

IFN-λ was found in the malignant bone marrow microenvironment, implying that IFN-λ may play a direct role on multiple myeloma development.

4.2 Role of endogenous IFN-λ in viral protection

The availability of IFN-λR1 knock-out mice allowed to investigate the role of type III IFNs *in vivo*. By using those mice, Mordstein and coll. showed for the first time the contribution of IFN-λ in the innate immunity against the influenza virus (Mordstein et al., 2008). Later, they found that IFN-λ played an important role in the defense against other pathogens that infect the respiratory tract, such as the respiratory syncitial virus, the metapneumovirus and the severe acute respiratory syndrome (SARS) coronavirus. However, the lassa fever virus which replicates in the liver, was not affected by the lack of IFN-λR1 (Mordstein et al., 2010). Although this study clearly demonstrated that IFN-λ played an important role in protecting the respiratory and gastrointestinal tracts against virus infection, in comparison with type I IFN, the protection provided by type III IFN remains limited. However, in combination, type I and type III may provide a better viral protection. When the response to both type I and type III is deficient, the mice are not able to clear the SARS coronavirus from the intestine as compared with mice in which type I or type III remains functional, implying that IFN-λ may strengthen the antiviral activity by acting as a first line of defense for the mucosa (Mordstein et al., 2008, 2010).

4.3 Clinical application of IFN-λ

The first use of IFN-λ in the clinic has started for hepatitis C. The phase 1b study has been conducted in patients with chronic genotype 1 hepatitis C virus infection ((HCV) (Muir et al., 2010)). Pegylated IFN-λ1 in combination or not with ribavirin (RBV, which belongs to a class of antiviral medications called the nucleoside analogues) has been used in this study to assess the efficacy and the potential cytotoxicity. The study was performed in 3 parts. The first part evaluated the pegylated IFN-λ as single agent for relapsed patients after IFN-α-based treatment. The second part concerned the combination of pegylated IFN-λ and RBV in treatment-relapse patients. The third part evaluated pegylated IFN-λ in combination with RBV in treatment-naïve patients. In addition, different doses (from 0.5 to 3 microg/kg) of pegylated IFN-λ were used. Fifty-six patients were enrolled. 24, 25 and 7 patients were used respectively for part 1 to 3. The data showed an antiviral activity in all doses of pegylated IFN-λ tested. 29% of treatment-naïve patients achieved rapid virological response. As expected, due to the limited IFN-λR1 distribution, the treatment was well tolerated with few adverse effects. Minimal flu-like symptoms and limited hematologic suppression were reported. In summary, the authors concluded that weekly PEG-IFN-λ with or without daily RBV for 4 weeks is associated with a clear antiviral activity in patients with chronic HCV. However, this study lacks a direct comparison between IFN-λ and IFN-α and the influence of viral and patient genotypes. Now it is well accepted that the response to IFN-α or the natural clearance of HCV infection is depending on single nucleotide polymorphisms (SNPs), upstream of IFN-λ3, that could be used as biomarkers to help determine the treatment outcome (Kelly et al., 2011). The first genome-wide association studies (GWAS) in HCV infection were reported by Ge and coll. They evaluated the treatment outcome in a group of 1671 patients of mixed ethnicity, receiving pegylated-IFN-α and ribavirin. An association was discovered between sustained viral response (SVR) to treatment and a cluster of seven SNPs linked to the IFN-λ3 gene, with the most significant SNP (rs12979860) demonstrating high statistical significance (Ge et al., 2009). Many other studies have

replicated these findings, demonstrating the high link between IFN-λ3 and treatment outcome (Labie and Gilgenkrantz, 2010; Mangia et al., 2010; McCarthy et al., 2010; Montes-Cano et al., 2010; Mosbruger et al., 2010; Rauch et al., 2010; Suppiah et al., 2009; Tanaka et al., 2009). However the mechanisms explaining this link remain to be determined. It is not clear yet if this SNP is associated with a constitutive production of IFN-λ that may play a role in HCV clearance and the success of IFN-α treatment. These results also suggest the therapeutic potential of the IFN-α and IFN-λ combination therapy as demonstrated for the hepatocellular carcinoma (HCC) mouse model (Lasfar et al., 2008).

5. Emergence of IFN-λ as a new antitumor agent

5.1 First report in animal model

Although they engage distinct receptors, IFN-α and IFN-λ induce similar cell signaling (Figure 1). Since IFN-α is widely used in clinic to treat cancer, we have investigated the potential antitumor activity of IFN-λ by using the mouse B16 melanoma model. We have chosen this cancer model because melanoma is a very aggressive cancer and one of the therapeutic agents frequently used in the treatment of melanoma is IFN-α. Significant improvements in relapse-free and overall survival, with postoperative adjuvant IFN-α therapy, have been reported by large and randomized studies (Kirkwood et al., 1996, 2001; Moschos et al., 2005). However, the beneficial effect of IFN-α was only obtained when the patients received high doses (20 MIU/m2 intravenously five times per week). Studies with low doses of IFN-α have not shown significant increase in overall survival (Cascinelli et al., 2001; Kleeberg et al., 2004). Usually, the dose for optimal antitumor activity is higher than the maximally tolerated dose. This dose-dilemma profoundly affects the acceptance of IFN-α treatment by both the clinicians and the patients. The adverse effects associated with high doses of IFN-α include myelosuppression and nervous system disorders. These effects often compromise the beneficial antitumor effect, with premature discontinuation of the treatment or the reduction of the dose of IFN-α. Since virtually all the cells of the body respond to IFN-α, it is not surprising that the patients develop numerous side effects. Making a dissection between the beneficial and harmful effects of IFN-α is a very challenging task, which requires more investigation of the interferon system. To investigate the antitumor effect of IFN-λ in melanoma, we used a gene therapy approach, consisting on the delivery of the IFN-λ gene to tumor cells. Gene transfer into tumor cells is a very useful approach to test the effectiveness of cytokines in animal cancer models. This approach does not require a production and the purification of the protein. The secretion of constant amounts of various cytokines by transduced tumor cells at the site of tumor growth could elicit more effective antitumor responses by acting directly on the tumor microenvironment. Another advantage of the cytokine gene transfer into tumor cells versus systemic administration is the potential of inducing the antitumor effect without eliciting the side effects associated with the systemic administration of high doses of cytokines.

To investigate the potential antitumoral role of IFN-λ, we first evaluated the response of B16 melanoma cells to IFN-λ, by analyzing STAT1 activation and MHC class I antigen expression. In comparison with IFN-α, we have found that IFN-λ induces weak STAT1 phosphorylation but strong stimulation of MHC class I antigen expression, indicating a difference between IFN-α and IFN-λ in the link intensity of cell signaling/biological activity (Figure 3). This result warrants further investigation, by comparing the response to IFN-α

and IFN-λ. As shown in this figure, although IFN-λ induces weak STAT1 activation, the biological activity can be very strong. By using gene transfer as illustrated in Figure 4, we next engineered B16 cells, which constitutively produced mIFN-λ (B16.IFN-λ cells). In response to their secretion of IFN-λ, B16.IFN-λ cells exhibited constitutively high levels of MHC class I antigen expression. All the C57BL/6 syngeneic mice injected with parental B16 cells developed tumors. However, the constitutive production of mIFN-λ by B16.IFN-λ cells markedly affected tumorigenicity of the cells. B16.IFN-λ cells were either rejected by the host or grew at a slower rate than control parental B16 cells. The antitumor effect of IFN-λ was dose dependent. B16.IFN-λ cells also inhibited the growth of parental B16 cells when both cell types were injected together (Lasfar et al., 2006). We also used B16.IFN-λ Res. cells which in addition to their constitutive IFN-λ secretion, are completely resistant to IFN-λ as demonstrated by the lack of IFN-λ–induced MHC class I antigen expression (Table 1). Interestingly, similar to B16.IFN-λ cells, we have found a reduction of the tumorigenicity of B16.IFN-λ Res. cells, implying the involvement of host antitumor mechanisms induced by IFN-λ.

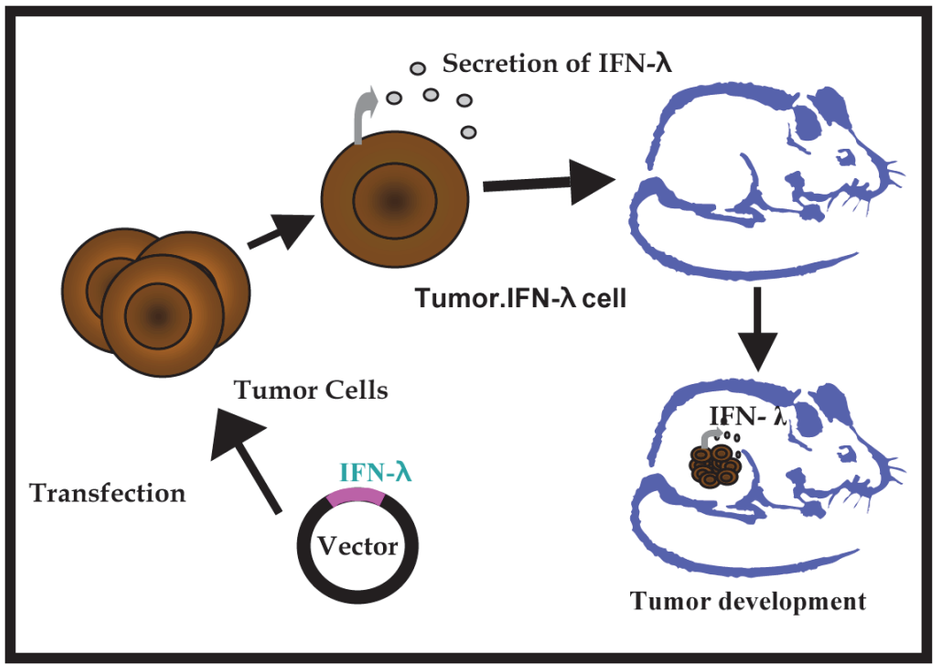

Fig. 4. IFN-λ gene transfer. Tumor cells were engineered to constitutively produce IFN-λ. Immunocompetent syngeneic mice were injected subcutaneously with the engineered tumor cells and monitored for tumor development.

Following our report on the characterization of the mouse IFN-λ system and the potent antitumor activity of IFN-λ in the B16 mouse melanoma model, independent groups confirmed the role of IFN-λ as antitumoral agent in melanoma and other tumor models. To demonstrate the antitumor activity of IFN-λ, Sato and coll. (Sato et al., 2006) used the mouse melanoma B16F0 and B16F10 and the Colon26 cell lines transfected with IFN-λ2 cDNA. The IFN-λ-transduced B16F0 cells showed an increased activity of caspase 3/7, an induction of p21 and a dephosphoryation of Rb, which triggered a cell cycle arrest and apoptosis. These

events obtained *in vitro*, were apparently associated with a tumor growth delay, observed *in vivo* after the injection of the B16F0 cells, transduced with IFN-λ. A delay in tumor growth was also observed after the administration of the Colon26 cells transduced with IFN-λ. By using the B16F10 cell line, which represents metastatic mouse melanoma cells, the authors showed that the overexpression of IFN-λ significantly inhibited lung metastasis. In another study, to evaluate the antitumor activity of IFN-λ, Numasaki and coll. (Numasaki et al., 2007) first transduced the mouse fibrosarcoma cells, MCA2005, with the retroviral vector PA317IL-28 (IFN-λ2). Following the injection of the engineered tumor cells to mice, the authors observed a significant antitumor and antimetastatic effect in mice inoculated with the MCA2005IL-28 in comparison with those injected with the parental tumor cells.

	IFN-λ production	Cell proliferation	MHC I antigen expression
B16 cells	-	++++	+
B16. IFN-λ cells	+ + + + + ++ +	+++	+ + + + + ++ +
B16. IFN-λ Res. cells	+ + + + + ++ +	+++	+

	Tumor necrosis	Inflammation	Tumor vascularization	Tumor mitotic rate
B16 cells	+ +	+/-	+ + + + + +	>14/HPF
B16. IFN-λ cells	+ + + + + +	-	+ +	5-10/HPF
B16. IFN-λ Res. cells	+ + + +	-	+ +	15/HPF
B16/B16. IFN-λ cells	+ + + + + +	-	+ +	3-5/HPF
B16/ B16. IFN-λ Res. cells	+ + + + + +	-	+ +	6-10/HPF

Table 1. Constitutive production of IFN-λ-induced tumor alteration. *In vitro* (Top) and *in vivo* (Bottom) analysis of the engineered B16 cells constitutively producing IFN-λ (B16.IFN-λ cells and B16.IFN-λ Res. cells). In contrast to the parental B16 and B16.IFN-λ cells, B16.IFN-λ Res. cells are completely resistant to IFN-λ, as indicated by MHC class I antigen expression unaffected by the presence of IFN-λ. HPF (High-Powered Field)

5.2 Investigation of the antitumor activities of IFN-λ in comparison with those of IFN-α in a BNL mouse model of hepatocellular carcinoma (HCC)

HCC is the most prevalent type of liver cancer. It is the fifth most common solid tumor and the third leading cause of cancer-related death worldwide. It is also the second most lethal cancer with the five-year survival rate below 9% (Farazi and DePinho, 2006; Lau and Lai, 2008; Sherman, 2005). Treatment options for HCC are limited mainly because of the inefficiency of existing anticancer chemotherapeutic drugs against HCC. Unfortunately, due to a lack of biomarkers and screening for HCC, most patients are diagnosed at advanced stages of the disease and do not meet strict selection criteria for potentially curative surgical tumor resection or orthotopic liver transplantation (OLT) (Mazzaferro et al., 2008, 2009; Taketomi et al., 2008) In patients with unresectable HCC and preserved liver function, transarterial chemoembolization (TACE) has been shown to prolong survival. However TACE is rarely curative, and progression-free survival beyond 24 months is not frequent (Georgiades et al., 2008; Lau and Lai, 2008). For patients with advanced disease, systemic chemotherapy is of limited benefit because of the resistance of HCC to existing

anticancer drugs and the fact that about 50% of patients with HCC die secondary to liver failure from cirrhosis (Di Bisceglie et al., 1998; Nagai and Sumino, 2008). HCC occurs most frequently in patients with cirrhosis as a result of chronic HBV and HCV infections, and alcohol abuse (El-Serag, 2002; Sherman, 2005). Although the link between the cancer and the viral infection is not fully understood yet, there is some suggestion that viral infection interferes with signal transduction and consequently, disrupts the normal, controlled growth of cells.

Since IFN-α is used in the clinic for the treatment of chronic HCV and HBV infections, several studies evaluated the effect of IFN treatment on the incidence of HCC (Sherman, 2005). It was previously shown that the systemic administration of high doses and long-term IFN-α into nude mice bearing human HCC with high metastatic potential, following curative resection, inhibited tumor metastasis and recurrence (Wang et al., 2003). The majority of clinical studies also concluded that IFN therapy, alone or in combination with ribavirin, decreased the incidence of HCC, particularly in patients with sustained virological response (Fattovich et al., 1998; Lin et al., 2007; Omata et al., 2005; Yu et al., 2006). Therefore, IFN alone or, perhaps, in combination with other drugs can be used as a preventive therapy against the development of HCC in HCV and HBV-infected patients. However, numerous side effects limit the overall tolerability of IFN-α, particularly in patients with cirrhosis (Llovet et al., 2000; Lo et al., 2007; Ueshima et al., 2008).

In the following part of this section, we describe our findings on the antitumor properties of IFN-λ on the BNL mouse model of HCC. To evaluate the antitumor activities of both IFN-λ and IFN-α, we used a gene therapy approach as previously described (Lasfar et al., 2006). We expressed IFN-λ and IFN-α genes under a strong constitutive promoter in BNL cells and selected stable cell lines, BNL-IFN-λ and BNL-IFN-α, constitutively expressing IFN-λ and IFN-α (Abushahba et al., 2010). Since the constitutive expression of IFN-λ at the tumor site was found to affect the tumorigenicity of B16 melanoma cells *in vivo* (Lasfar et al., 2006), we examined whether similar effects of IFN-λ would be displayed in the case of BNL hepatoma. Mice injected with BNL.vector or parental BNL cells developed tumors in 4 to 6 weeks, whereas the tumor appearance for BNL.IFN-λ cells was significantly delayed. Similar effects were obtained in mice inoculated with BNL.IFN-α cells. These experiments demonstrated that constitutive expression of IFNs at the tumor site resulted in the delay of tumor growth *in vivo*. Interestingly, we found that IFN-α and IFN-λ exhibited similar antitumor activities (Abushahba et al., 2010).

6. Antitumor mechanisms of IFN-α and IFN-λ

6.1 Antitumor mechanisms of IFN-α

Despite the antiproliferative effects of IFN-α on cells, it seems that the direct effects on tumor cells may not be the major mechanism by which IFN-α displays its antitumor activity. IFN-α can act indirectly on the tumor by inhibiting angiogenesis which is induced by the tumors and is required to promote their growth and metastasis (Sidky and Borden, 1987). In mice bearing human tumors, it was clearly demonstrated that the antitumor activity of IFN-α is associated with the inhibition of tumor angiogenesis in bladder carcinoma (Dinney et al., 1998) and prostate cancer (Dong et al., 1999). The involvement of the immune system in the antitumor mechanism of IFN-α was strongly suggested by Gresser and coll. Early

studies in tumor models have shown that an intact immune system was essential in IFN-α-induced antitumor activities. The inhibition of FLC (Friend Leukemia Cells) by IFN-α in mice was shown to depend on the activation of host cells, such as NK cells and macrophages (Gresser et al., 1994). Both host humoral and cellular immune mechanisms were involved in the continued suppression of Friend erythroleukemia metastases after IFN-α treatment in mice (Gresser, 1991). In addition, effective adaptive immunotherapy was observed in a T-cell lymphoma model, after the injection of tumor-sensitized spleen cells and IFN-α. By using antibodies against different immune cell populations, it has been shown that CD4+ T lymphocytes and CD8+ T lymphocytes were the major effectors in the antitumor activities induced by IFN-α (Kaido et al., 1994; 1995).

6.2 Antitumor mechanism of IFN-λ

Although IFN-α and IFN-λ signal quite similarly (Figure 1), the mechanisms underlying the antitumor activity of IFN-λ may be qualitatively different from IFN-α. As previously described, we initially investigated whether type III IFNs also possessed antitumor activities utilizing a gene therapy approach in the B16 melanoma model. B16 melanoma cells that were engineered to constitutively secrete IFN-λ and were either sensitive or resistant to IFN-λ, were transplanted into two groups of mice. Potent antitumoral activity was observed for both groups of mice. Since secreted IFN-λ did not affect the proliferation rate of B16 melanoma cells *in vitro*, studies in the B16 melanoma model suggested that IFN-λ acted through host mechanisms to elicit its antitumor activity (Lasfar et al., 2006). However, we did not observe a significant long-lasting immunity, implying that there may be a lack of effective adaptive immunity in the mice which rejected the tumor. On the other hand, we noticed a reduction in tumor vascularity in the presence of IFN-λ, suggesting a potential role of IFN-λ in the tumor microenvironment (Lasfar et al., 2006). Since we found that keratinocytes are highly sensitive to IFN-λ and they are known to interact with melanocytes, the cells from which the melanoma originates, we suggested that IFN-λ delivery to the tumor microenvironment may affect the function of the keratinocytes as well as other stroma cells thereby promoting inhibition of tumor growth (Lasfar et al., 2006) (Figure 5).

Natural Killer (NK) cells, the major effectors of innate immunity, could also be recruited to the tumor microenvironment and help destroy the tumor cells. Two groups have reported that NK cells played a role in the antitumor mechanisms of IFN-λ. Sato and coll. (Sato et al., 2006) have described the involvement of NK cells in melanoma and colon cancer antitumor responses. They have shown that transient transduction of B16 cells with mouse IFN-λ cDNA, enhanced MHC class I and Fas expression, suppressed cell proliferation by inducing increased caspase-3/7 activity and increased $p21^{Waf1/Cip1}$ levels and dephosphorylated Rb (Ser^{780}) *in vitro* (Sato et al., 2006). This meant that IFN-λ was able to induce cell cycle arrest and apoptotic cell death *in vitro*. In addition, they have demonstrated that overexpression of IFN-λ inhibited local and pulmonary metastatic tumor formation *in vivo*. Depletion of NK cells, by injecting an anti-asialo GM1 antibody before tumor cells injection, revealed that NK cells are important in this IFN-λ-mediated tumor growth inhibition *in vivo*, suggesting that IFN-λ activated the innate immune response (Sato et al., 2006). Numasaki and coll. (Numasaki et al., 2007) have also implicated NK cells, polymorphonuclear neutrophils and $CD8^+$ T cells in the antitumoral activity induced by IFN-λ in the MCA205 murine fibrosarcoma mouse model. Inoculation of MCA205-IFN-λMCA205-IFN-λ cells, into mice

enhanced IFN-γ production and cytotoxic T cell activity in the spleen. The antitumor activity of IFN-λ was partially dependent on IFN-γ. In addition, IFN-λ increased the total number of splenic NK cells in severe combined immunodeficiency (SCID) mice, enhanced IL-12-induced IFN-γ production *in vivo,* and expanded spleen cells in C57BL/6 mice. Furthermore, they reported that IL-12 augmented the IFN-λ-mediated antitumor activity in the presence or absence of IFN-γ. Based on their findings, they suggested that IFN-λ is able to induce both innate and adaptive immune responses to suppress *in vivo* tumor growth (Numasaki et al., 2007).

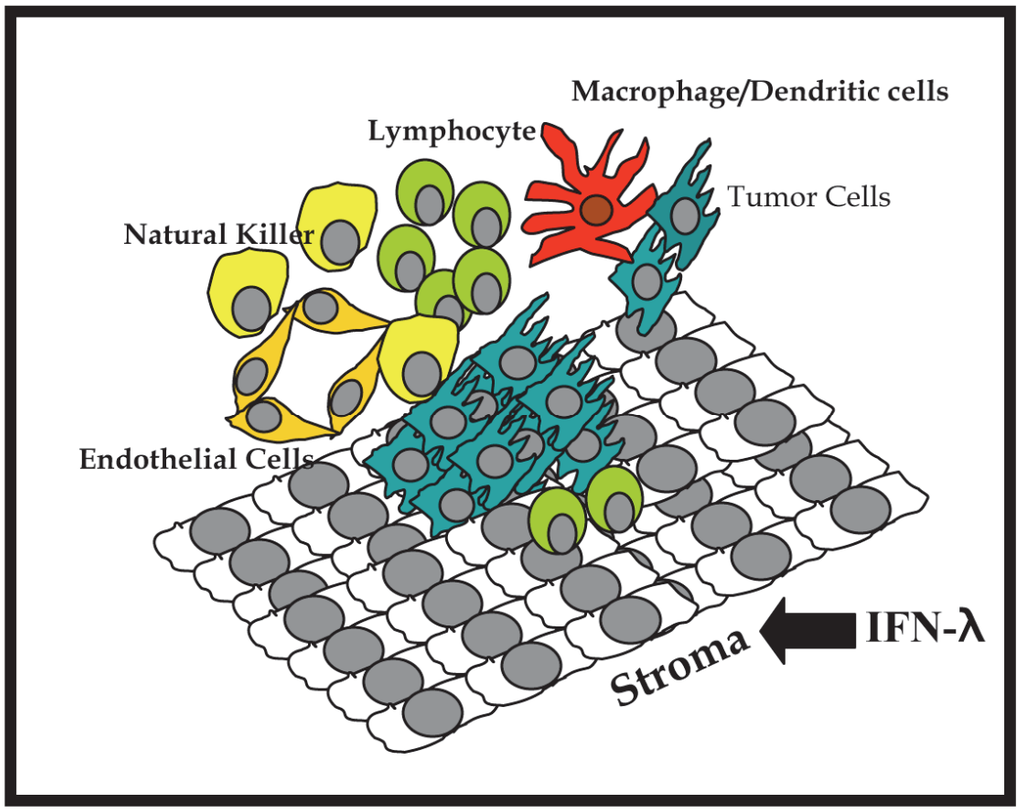

Fig. 5. Potential role of IFN-λ in the tumor, microenvironment. The IFN-λ delivered to the tumor microenvironment may induce its potent antitumor activity by modulating the interaction between the tumor cells and the normal cells, including the recruited immune cells.

Our recent study in the BNL hepatoma model also revealed that NK cells are implicated in the antitumor activity induced by IFN-λ and probably more potently than in the antitumor activity induced by IFN-α. However, in contrast to IFN-α, we did not detect any response after *in vitro* treatment of NK cells by IFN-λ, suggesting that IFN-λ may activate other cells, which then mediate NK cell activation (Abushahba et al., 2010). There was also a marked NK cell infiltration in IFN-λ producing tumors. In addition, IFN-λ and, to a lesser extent, IFN-α enhanced immunocytotoxicity of splenocytes primed with irradiated BNL cells. Splenocyte cytotoxicity against BNL cells was dependent on IL-12 and IFN-γ, and mediated

by dendritic cells. In contrast to NK cells, isolated from spleen, CD11c+ and mPDCA+ dendritic cells responded directly to IFN-λ, suggesting that the effects of IFN-λ on NK cells are mediated by other IFN-λ-responsive cells, such as DCs (Abushahba et al., 2010). On the other hand, a significant decrease in CD4+CD25+Foxp3+ Tregs was observed in mice inoculated with BNL cells secreting IFN-α, whereas the moderate decrease in Tregs observed in mice receiving BNL cells secreting IFN-λ was not statistically significant (Abushahba et al., 2010). Therefore, antitumor mechanisms activated by IFN-α and IFN-λ may differ; IFN-λ increased the number of NK cells at the tumor site whereas IFN-α had a stronger effect on Tregs in the BNL model.

These studies altogether suggest that although IFN-α and IFN-λ signal quite similarly (Figure 1), differences exist in their biological potency, kinetics and the sets of target cells sensitive to IFN-λ and IFN-α. Therefore, these two types of IFNs may have distinct physiological functions.

7. IFN-λ as a potential ally rather than alternative to IFN-α

Unlike IFN-α, only a small subset of cells are sensitive to IFN-λ, implying that its potential clinical use may be associated with limited side effects. This presumption raises the question whether IFN-λ could be an alternative to IFN-α in cancer therapy. However, despite the severe and numerous side effects inherent to IFN-α treatment (Moschos et al., 2005), we believe that alternative treatment to IFN-α should be weighed first against the real benefits to patients in terms of overall survival and their tumor clearance. We have, demonstrated in the BNL hepatoma model that the combination of IFN-λ and IFN-α could achieve a stronger antitumor activity in comparison with the use of each IFN alone (Lasfar et al., 2008). The benefits of the combination therapy of IFN-λ and IFN-α have been demonstrated by both a gene therapy approach and direct administration of IFNs to the mice bearing the tumors. The mice injected with BNL cells secreting both IFN-λ and IFN-α can completely reject the tumor, in contrast to the mice that only received the BNL.IFN-λ cells or the BNL.IFN-α cells. Furthermore, mice bearing established tumors and treated with exogenous IFN-λ and IFN-α, showed a drastic tumor repression. This effect was observed when the IFNs were delivered locally and even at low doses. Therefore, we believe that IFN-λ is not simply acting like IFN-α, with reduced side effects, but can be combined with IFN-α to achieve efficient antitumor activity. Combination of IFN-λ with low doses of IFN-α, which are sub-therapeutic but less toxic (Kleeberg et al., 2004), may improve IFN therapy and benefit cancer patients. Combinational therapy of IFN-λ and IFN-α may achieve ultimate antitumor activity by inducing complementary mechanisms directly on the tumor cells or by indirectly modulating the tumor microenvironment, thereby leading to the stimulation of the immune response against the tumor and the inhibition of tumor angiogenesis. By acting with different intensities on the same targets, IFN-λ and IFN-α may generate a high level of synergy, leading to a potent antitumor activity.

8. Conclusion

Similarly to IFN-α, IFN-λ has been shown to play an important role in cancer and viral disease treatment. Although the two IFNs act through an identical signaling pathway in the cell, the pattern of their activity seems to be different *in vivo*, implying that IFN-λ and IFN-α

are not redundant cytokines. By acting on some targets with different intensity, we believe that IFN-λ and IFN-α act in concert to better control tumor development *in vivo*. Therefore, to achieve better treatments for viral diseases or cancers, we believe that the development of a combination therapy rather than the use of each IFN alone will be more beneficial for the patients. The combination of IFNs with other cytokines, growth factors, or their antagonists could also be a viable strategy for the improvement of the IFN therapy. Transforming Growth Factor-beta (TGFβ) which plays a dual role in cancer, mediating tumor suppresive activities at early stages and prooncogenic activities at later stages of tumor progression (Javelaud et al., 2008), could represent one potentially important modulator or mediator of the IFN response. In different cancers, inculding melanoma, several cellular pathways modulate the activity of TGFβ (Lasfar and Cohen-Solal, 2010). Understanding the potential crosstalks between IFN-α, IFN-λ and other cytokines or growth factors, such as TGFβ, could be rewarding and lead to new preclinical studies in animal models and new clinicals trials resulting in better cancer treatments.

9. Acknowledgment

We thank Dr. Sergei Kotenko for his valuable support and useful discussions. We also thank Dr. Andrew de la Torre and Dr. Walid Abushahba for their input in the hepatoma antitumor studies. We are grateful for the direct or indirect help of Dr. Ion Gresser, Dr. Jeanne Wietzerbin, Dr. Christian Billard, Dr. Jean-Pierre Kolb, Dr. Michael Tovey and Dr. Sidney Pestka.

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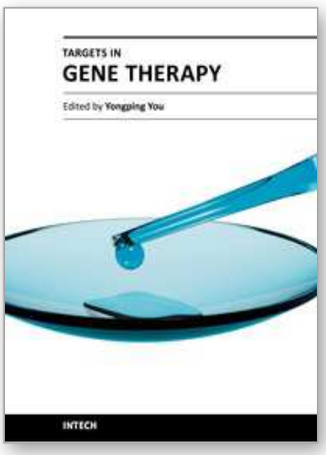

Targets in Gene Therapy

Edited by Prof. Yongping You

ISBN 978-953-307-540-2
Hard cover, 436 pages
Publisher InTech
Published online 23, August, 2011
Published in print edition August, 2011

This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ahmed Lasfar and Karine A. Cohen-Solal (2011). Emergence of IFN-lambda as a Potential Antitumor Agent, Targets in Gene Therapy, Prof. Yongping You (Ed.), ISBN: 978-953-307-540-2, InTech, Available from: http://www.intechopen.com/books/targets-in-gene-therapy/emergence-of-ifn-lambda-as-a-potential-antitumor-agent

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